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STRUCTURE OF SPORE WALL IN GANODERMA

LESLIE C. COLEMAN

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(WITH PLATE V)

In the classification of the Polyporaceae, spore characters have usually received but little attention. This is due largely to the fact that in many forms spores have not as yet been observed. An incorrect interpretation of spore structure in certain forms which have been variously placed in the genera *Fomes*, *Ganoderma*, and *Elfvingia*, however, has partially been responsible for the failure to use what is undoubtedly a valuable diagnostic character. The present investigation has been undertaken in an attempt to clear up the misconceptions which have arisen in this regard.

The genus *Ganoderma* was established by KARSTEN (7) as including only one species, *G. lucidum*, characterized by having the pileus covered by a shiny crust, and the spores ovate or elliptical, warty, and yellowish brown in color. Subsequent systematic writers, like PATOUILLARD (10) and MURRILL (9), have retained the genus *Ganoderma* but on the basis of the shiny crust alone. Others, like SACCARDO (11), have given it only subgeneric rank. The warty character of the spore coat has been considered by them all as of subordinate importance. PATOUILLARD, it is true, has used this character in subdividing his genus *Ganoderma*, but the other writers have noted it only in specific descriptions. In fact, MURRILL places many of the forms which show quite definite affinities with *G. lucidum* as to spore characters in KARSTEN's genus *Elfvingia*, which indicates the small importance he attaches to such characters.

All of the writers just cited, as well as others, as a matter of fact have misinterpreted the character of the spore wall. This was first pointed out by ATKINSON (2, 3) in two papers which appeared in 1908. He showed that the outer surface of the spore wall in a number of forms examined by him is smooth, not echinulate or verrucose as had been stated previously. In the first of the two papers he states:

> The spore wall is hyaline or nearly so and is perforated by numerous slender rod-like extensions of a brown or yellowish brown substance which appear as

if they were projections of the colored contents of the spore. These do not extend beyond the outer surface of the wall, and they radiate from the endospore through the hyaline wall. They are especially prominent at the smaller end of the oval spore where the hyaline wall is considerably thicker, sometimes forming a broad cone-like cap to the spore.

In his second paper, dealing with *Ganoderma applanatum* Pers., he states:

> Not only is the spore wall hyaline or nearly so, it appears to be of a less firm consistency than the dark rods or perforating substance. In some cases, perhaps due to a certain age of the spore wall, when its consistency is less firm than at other times, the spore wall collapses to a certain extent and there is a tendency for the hyaline part of the wall to collapse between these dark areas, thus giving a roughened or slightly echinulate appearance to the spore.

In another part of the same paper he states, "The hyaline or nearly hyaline wall is perforated with numerous short dark lines or plugs which radiate from the spore content or endospore membrane through the epispore and end even with its external surface."

It will be seen that Atkinson was in doubt as to the real nature of the "dark rods" to which he ascribed the echinulate appearance in surface views of the spore. It is interesting in this connection to note that Patouillard in 1899 had observed that the spore wall in the forms studied by him was differentiated into distinct epispore and endospore. He states:

> Elles sont formées d'une membrane interne épaisse et colorée en brune ou jaune plus ou moins foncé. Sur cette membrane on observe souvent des petites verrues serrées; l'épispore est mince, incolore et se moule exactement sur les asperités de l'endospore, c'est elle qui est échancrée et forme une pointe incolore à la base de la spore.

Miss Ames (1), working in Atkinson's laboratory, has confirmed Atkinson's description of the spore wall structure. She lists sixteen species of *Ganoderma* examined, and states that they are all characterized by having spore walls smooth, with dark lines extending into the hyaline or nearly hyaline wall from the darker spore content.

White (15) gives a decidedly different interpretation to the structure of the spore wall in *Fomes applanatus*. He states:

> In studying its development we find that the basidiospore starts out with a hyaline wall and then that later within this outer thin walled basidiospore a

rough-coated thick and yellow walled endospore is formed. The spore "wall" in one sense then is accordingly double. As the endospore is more shortly elliptical than the original basidiospore, the tip of the latter is not occupied, and this hyaline tip being thin walled and without supporting contents usually collapses. This gives to the mature spore the "truncated" appearance so invariably noted.

Finally, BULLER (4) disagrees with WHITE'S interpretation, and in general confirms ATKINSON'S observations. He states:

It seems to me that each spore has a continuous rather thick wall made up of two layers: (1) an outer very thin, colorless, homogeneous layer formed whilst the spore is growing from a tiny rudiment to full size, and (2) an inner and a much thicker layer formed more slowly during the ripening of the spore, of a whitish or yellowish color and marked from within outwards by numerous fine yellowish brown striae. What the nature of these striae is I do not know, but I do not regard them as papillae.

From these citations it will be seen that the structure of the spore wall in these interesting forms is still a matter of uncertainty. This is hardly to be wondered at when we consider that apparently all the observations so far have been made on intact spores, either in collected spore material or in hand sections of the sporophore unstained in any way. When we remember that the spore wall is scarcely 0.5 μ thick, the difficulty of making definite observations will be understood. When, therefore, Dr. FAULL suggested to me the advisability of making a more thorough study of the subject, I gladly availed myself of the opportunity so generously furnished by him. He placed at my disposal excellent material of a number of forms. To this has been added a huge mass of spore material of *Ganoderma* (*Fomes*) *applanatum* kindly given me by Dr. J. H. WHITE.

Material and methods

The basis of this study has been material of *Ganoderma applanatum* and *G. tsugae;* of the former I have had abundant spore material as well as sporophore material fixed in picro-sublimate; of the latter I have had good wet material in alcohol. In addition to this I have studied wet material of *G. tornatum* and *G. Lionettii*, spore material and dry sporophore material of *G. lobatum*. For comparison, material of a distinctly different form, *Fomes fomentarius*, was given to me by Dr. FAULL both as imbedded and spore material, and has been included in the study. Miss AMES included this form in the

genus *Ganoderma* on the ground of spore characters. As a matter of fact the spores are of a very different type, so much so that it seems impossible that she could have observed them at all. Previous microscopic studies of *Ganoderma lucidum* had verified the conclusions of ATKINSON (2) so far as the similarity of the spore wall in this form to that in the other forms mentioned is concerned.

As the spore coat is hard, the material for critical study was imbedded in paraffin of 60° melting point, and for most of the sectioning resort was had to the cooling action of an ether spray, a freezing attachment for the microtome not being available. As the spores of *G. applanatum* average about 5 μ in thickness, those of *G. tsugae* being somewhat larger, sections were cut down to 2 μ in thickness.

In trials to obtain a differential staining of what seemed to be two parts of the spore wall, the best results were obtained by the use of safranin and Licht Grün. Each of these if used separately was found to stain both parts of the spore wall, the endospore taking up the stain more strongly. A beautiful differential staining, however, was obtained when the sections were kept in the safranin bath (1 per cent in 50 per cent alcohol) from 12 to 24 hours, run quickly up through the various grades of alcohol, and kept in the Licht Grün bath (0.5 per cent in 90 per cent alcohol) for from 10 seconds to 1 minute, and then rapidly dehydrated and passed into xylol. This was used as the regular method of staining throughout the investigation.

Mature spores

In thin sections of the sporophore mature spores could be found in abundance, and many of them could be studied in section. *G. tsugae* proved on the whole to be more satisfactory, as its spores are larger than those of *G. applanatum*. The microscopic examination was made with a Zeiss apochromatic 1.5 mm. objective with compensation ocular no. 8. Higher power oculars (Zeiss nos. 12 and 18) were tried, but it was found that they did not give a satisfactory definition.

The microscopic picture, as found in the case of many hundreds of spores examined, showed quite definitely that the interpretation of ATKINSON, Miss AMES, and BULLER is incorrect, while that of

WHITE in the main represents the actual structure of the wall. The hyaline epispore with its conspicuous thickening at the narrower distal end takes on a beautiful green color, the Licht Grün having removed all the safranin in this area. On the other hand, the thicker endospore retains the safranin very strongly where the counter staining had not been too prolonged. The spore content, for the most part, takes on a much lighter stain than the endospore, the nucleus being visible as a more deeply stained mass in many of the sections. From the endospore project fine processes or spines, which are somewhat thicker at the inner end out into the epispore. These projections appear in some cases not to extend quite to the outer surface of the epispore, although I am not prepared to speak definitely on this point. These projections or spines show a red stain uniform with that of the endospore, and appear quite definitely as outgrowths from it. They are not rodlike extensions of the spore contents, as ATKINSON and Miss AMES believed, nor are they striae extending through the endospore wall as indicated by BULLER. These projections are frequently to be found, especially in *G. tsugae*, beautifully defined in the hyaline cap or beak. Here they show as definite red lines against the greenly stained groundwork of the beak.

A further consideration of this hyaline cap or beak is necessary. WHITE pictures it as empty. He uses the term "not occupied," and accounts for its collapse, which is so frequently to be seen in mature spores, on this supposition. The whole beak, however, even in the thinnest sections, is stained uniformly green, which would not be the case of course were there a space between the endospore and the epispore in this region.

Figs. 1 and 3 show the structure of the spore wall in *G. tsugae* and *G. applanatum* respectively, the endospore being marked in black and the spore contents being omitted. In addition to the points just mentioned, fig. 1 brings out another interesting feature of the spore wall, which ATKINSON has already noted, but which the microphotographs accompanying his papers do not illustrate; that is, the fact that at the proximal end of the spore there is a small pore in the endospore which is somewhat asymmetrically placed. Around this pore the endospore projects outward, almost if not

quite to the outer surface of the epispore. This, as ATKINSON stated, marks the point of attachment of the spore to the sterigma. The view expressed in most systematic works on these forms, that the spore is attached to the sterigma by its hyaline or (as is frequently stated) truncate narrower end, has been disproved by ATKINSON, WHITE, and BULLER.

Figs. 2, 4, and 5 give similar sectional views of the spores of *G. lobatum*, *G. tornatum*, and *G. Lionettii* respectively. The structure of the spore wall in these forms is seen to be similar in all essential respects to that found in *G. tsugae* and *G. applanatum*. In the case of the last two forms, however, there were no projections of the endospore out into the hyaline beak, although the beak itself takes the Licht Grün stain even more strongly than in the other forms.

It may perhaps be wondered how such able observers as ATKINSON and BULLER could have misinterpreted the structure of the spore wall. It is quite explicable when we consider that the endospore, although yellowish brown in color, is quite transparent. Thus when the microscope is focused in the median plane of the spore, the projections above and beneath appear in the picture even under the highest magnifications and give the optical effect of striations. Certainty as to the structure can be attained only after a study of many very thin spore sections, something which I believe neither ATKINSON nor BULLER made.

Reference has already been made to the observations of ATKINSON on the collapse or shrinkage of the hyaline epispore, while, as WHITE has noted, the collapse of the hyaline beak in *G. applanatum* is a phenomenon very generally observed. As a matter of fact, in all dry material most mature spores show this collapse so markedly that in systematic works the spores of these forms are generally described as "truncate at the base." In the case of two of the forms studied, *G. tornatum* and *G. Lionettii*, where the long hyaline beak is not supported by any projections of the endospore, the beak apparently does not collapse, but instead breaks off. As to the collapse at other regions of the spore wall, I have not seen any evidence of the epispore becoming molded on to the echinulate surface of the endospore, as described by PATOUILLARD and indicated by ATKINSON. Commonly, however, the epispore seems to have shrunk so as

to have its outer surface resting on the ends of the spines of the endospore. The variation in thickness of the episore is no doubt due to the presence of varying quantities of water. This question will be discussed later in dealing with the chemical nature of the spore wall.

Development

BULLER has already indicated the general course of development of the spore wall. He states that the endospore begins to make its appearance only during the ripening of the spore, that is, after the spore has attained its full size. A careful study of developmental stages in *G. applanatum* has shown that, while there is no sign of endospore formation until after the spore has taken on its definitive shape, measurements indicate that the spore continues to increase in size after this process has begun. Figs. 7–13 show stages in spore development with special reference to the differentiation of the spore wall. In the stage illustrated in fig. 7, the young half-grown spores were found stained almost entirely green after the safranin Licht Grün treatment. A few coarse granules were to be found scattered irregularly in the cytoplasm, which had retained the safranin stain, but these bore no demonstrable relationship to the cell wall. In this stage the nuclei had not yet migrated from the basidium.

Fig. 8, showing in section two basidiospores, illustrates what must be considered as the earliest demonstrable stage of endospore formation. Here we have the epispore and cytoplasm stained green. In addition there is a line of rather coarse granules which have retained the red stain, and which appear just inside the spore surface. At the apex of the spore, they have been laid down at a distance from the point. Between them and the surface is a distinct area free from granules which represents the epispore of the mature spore. A similar stage is illustrated in figs. 9 and 11, where a spore is shown in longitudinal and transverse section respectively.

In figs. 10 and 12 a later stage is shown. The granules have fused into a continuous membrane, from which short projections have grown out toward the epispore surface. It seems highly probable that up to this stage, and in fact even later, the limiting membrane of the spore has not yet assumed the character of a definite wall, although there has been a differentiation of a hyaline layer. The outgrowth of spiny projections from the endospore, which finally

reach almost or quite to the outer surface of the spore, would be difficult to understand if a definite epispore had already been formed. On the other hand, the presence of a plastic episporic area bounded by an outer limiting membrane would allow for this outgrowth without any difficulty. WHITE's conception of an endospore quite separate from the epispore is incorrect, I think. It is true that spores and fragments of spores can frequently be found which show portions of the epispore separated from the endospore, but this is probably a tearing along the line of demarcation between differently organized layers of a single wall, rather than a separation of two distinct walls.

Chemical composition

To verify more fully the conclusions reached as a result of microscopic examination, and to ascertain if possible the chemical nature of the episporic and endosporic layers, I subjected the wall to microchemical tests. The work of GILSON (5), WINTERSTEIN (16), and VAN WISSELINGH (13) has shown that the cell wall in most fungi does not contain cellulose. VAN WISSELINGH has further shown that its place is taken for the most part by chitin. Repeated and prolonged tests made on the spores and sections of sporophores of *G. applanatum*, *G. tsugae*, and *G. tornatum* with chlorzinciodine, iodine in potassium iodide with dilute H_2SO_4, and cuprammonium oxide gave no evidence of cellulose, either in the spore wall or in the mycelium of the sporophore. Both chlorzinciodine and cuprammonium oxide produced a distinct swelling of the epispore, but it did not lead to its separation from the endospore layer. HANSEN (6), in his study of the spores of *Coprinus stercorarius*, has recorded a similar swelling of the epispore in that form, when the spores were treated with chlorzinciodine, which in the case of that form led to the separation of the two layers.

Tests for chitin were carried out according to VAN WISSELINGH's method and VOUK's (14) modification. Of the two, VAN WISSELINGH's original method was found much more satisfactory. It consists in heating the material to be examined in concentrated KOH in a sealed tube to a temperature of 160°–180° C., which changes the chitin to chitosan, washing with 90 per cent alcohol, and then testing on the slide with dilute iodine in potassium iodide to which a drop of dilute H_2SO_4 is added. The chitosan gives a very character-

istic reddish violet color reaction when treated in this way. Dry spore material of *G. applanatum*, celloidin sections of the same species, and hand sections of *G. tsugae* were used for the purpose. After heating, the KOH solution was diluted with distilled water to enable centrifuging, the material separated in a centrifuge, and washed with 90 per cent alcohol. The spores and shreds of sections remaining after this treatment were then treated on the slide. It was found in the case of spore material that, in the final centrifuging with alcohol, the whole mass distributed itself on the sides of the tube and stuck there as if it were gummed, and could not be dislodged by the centrifuging process.

An examination of the spore material after treatment with the coloring reagent showed both epispore and endospore intact. The cell contents had entirely disappeared, of course. The epispore remained quite hyaline, while the endospore had taken a beautiful reddish violet color. Figs. 13 and 14 show two spores thus treated in optical section, the blackened portion of the wall representing the reddish violet endospore. As will be noted in comparing these figures with fig. 3, there has been a slight swelling of both epispore and endospore, with a shortening of the spiny processes on the latter, so as to separate them distinctly from the surface of the spore. The results obtained in this test show that ATKINSON's conception of the spore wall is erroneous, and that the striae observed by BULLER are an optical illusion.

It appears certain that the endospore does not consist entirely of chitin. According to VAN WISSELINGH, chitosan dissolves in dilute HCl, and an attempt to dissolve out the endospore after treatment with KOH failed even when the slide was heated. We must conclude, therefore, that the endospore consists of chitin and some other compound or compounds at present unknown.

The study of the epispore was more difficult and puzzling. I was inclined to believe that this portion of the wall contains a gum or slime. The presence of such a substance seemed probable from the fact that spores caught on sporetraps made of microscopic slides adhere very firmly, and can be washed off only with difficulty. The epispore, however, could not consist entirely or even mainly of a gum, as it does not swell up very appreciably when the spores are

kept in water even for long periods. I have not been able to obtain fresh spores for examination, however, and they might conceivably give somewhat different results from spores which have been kept for several years on a glass slide.

I attempted to obtain a differential stain of the epispore with Ruthenium red, which according to MANGIN (8) is an extremely valuable differentiating stain for pectin bodies and the gums and slimes derived from them. While a stain of any kind is admittedly a very unsafe means of distinguishing groups of chemical compounds, it was thought that the use of this one might throw light on the question of the composition of the epispore. Spores of *G. applanatum* stained with Ruthenium red (1 part in 5000) undoubtedly give a color in the episporic region, but the endospore and also the spore contents take up the stain. It is obvious therefore that this is not a specific stain for these bodies; in fact, TOBLER (12) has already shown that other bodies, such as glycogen, take up the stain strongly.

Results from treating sections of the sporophore of *G. tornatum* were even less satisfactory. The very pronounced hyaline beak of the spore remained unstained, while the endospore and spore content became colored. The hymenial layer also appeared red, while the brown trama remained unstained. The presence of glycogen would account for the staining of the hymenial layer and the spore contents, but not for that of the endospore.

Finally I attempted to dissolve the epispore, leaving the endospore intact. For the purpose, following VAN WISSELINGH, spore material of *G. applanatum* was heated in glycerin to 290° C. (the boiling point of glycerin). In this way evidence was obtained of the solution of the epispore, but portions of it usually remained. Heating in dilute H_2SO_4 gave much better results. Spore material of both *G. applanatum* and *G. tornatum* heated on the slide in from 5 to 20 per cent H_2SO_4 gave large numbers in which the epispore had entirely disappeared, leaving the endospore intact. This reaction indicates that the epispore has the characteristics of a hemicellulose, although it gives no color reaction with chlorzinciodine or with iodine and dilute H_2SO_4. In any case its chemical composition is obviously very distinct from that of the endosporic layer of the spore wall.

Conclusions

My conception of the spore wall structure in these forms is as follows. The epispore represents the primitive spore wall, and is probably comparable with the undifferentiated spore wall of such a form as *Fomes fomentarius*, which is very thin, and which, treated by the same methods used for *Ganoderma* spores, has shown no differentiation whatever. It consists of a hemicellulose with possibly a gum, which latter, if present, functions in attaching the spore to the surface upon which it falls. The endospore is composed of chitin and other compound or compounds. It is laid down on the inner margin of the epispore as a series of granules which later fuse to form a membrane. This thickens and develops on its outer surface spiny processes which project into the epispore at a time when the latter is still plastic. The whole endospore structure obviously functions as a sort of skeletal support to the thin and collapsible primary spore wall.

It seems probable that this highly specialized structure has to do with preserving the spore through unfavorable seasons. It is a fact that the spores of both *G. applanatum* and *G. lucidum* are extremely difficult to germinate under laboratory conditions. WHITE, in his work on the former species, was rarely able to obtain germination, and could not establish the factors which governed it. My work on *G. lucidum* was even less successful, for, although attempted repeatedly, I have never succeeded in obtaining a single germination. If we take a thin walled spore such as that of *Fomes fomentarius*, germination of fresh material is readily to be obtained according to FAULL, who has made a thorough study of the subject.

Possibly the fact that *G. applanatum*, and presumably other species of *Ganoderma*, discharge spores over long periods during which conditions for germination cannot always be favorable, while *Fomes fomentarius* discharges only during a comparatively short period in the spring, may bear some relation to the differing structure of the spore wall.

The peculiar structure of the spore wall described in this paper is characteristic of species found in such widely separated areas as America, Europe, and India. It would seem to be a character of much greater importance from a systematic standpoint than many

of those at present being used in the classification of the Polyporaceae. A revision of the genera *Fomes*, *Ganoderma*, and *Elfvingia*, which in standard systematic works are given as containing species with the spore characters here described, together with other forms showing quite different spore characters, seems to be urgently required if our classification is to represent real relationships.

As indicated by the title of this paper, I consider that all forms showing the spore characters described should be brought together under the genus *Ganoderma* Karst.

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EXPLANATION OF PLATE V

All figures have been drawn with the aid of an Abbé camera lucida, using Zeiss apochrematic 1.5 mm. objective, N. ap. 1.30 mm. and compensating ocular no. 8. They represent a magnification of approximately 2000 diameters.

FIG. 1.—Basidiospore of *Ganoderma tsugae* in longitudinal section.

FIG. 2.—Basidiospore of *G. lobatum* in longitudinal section.

FIG. 3.—Basidiospore of *G. applanatum* in longitudinal section.

FIG. 4.—Basidiospore of *G. tornatum* in longitudinal section.

FIG. 5.—Basidiospore of *G. Lionettii* in longitudinal section.

FIG. 6.—Basidium of *G. applanatum* showing first signs of basidiospore formation on sterigma to left; section fixed in picrosublimate and stained with safranin and Licht Grün (same process used for figs. 7–12).

FIG. 7.—Longitudinal section of basidium of *G. applanatum*, showing three basidiospores about half developed; larger dots represent granules stained red, rest of the spore cytoplasm being stained green.

FIG. 8.—Longitudinal section of basidium of *G. applanatum*, showing basidiospores which have assumed their definitive shape but not their full size; first signs of endospore shown as a line of granules stained red inside the epispore.

FIG. 9.—Longitudinal section of basidiospore of *G. applanatum* which has become detached from the sterigma, showing about same stage as in fig. 8.

FIG. 10.—Longitudinal section of basidiospore of *G. applanatum*, showing endospore granules fused into continuous wall and spiny projections beginning to develop.

FIG. 11.—Transverse section of basidiospore of *G. applanatum*, showing same stage of endospore development as in fig. 9.

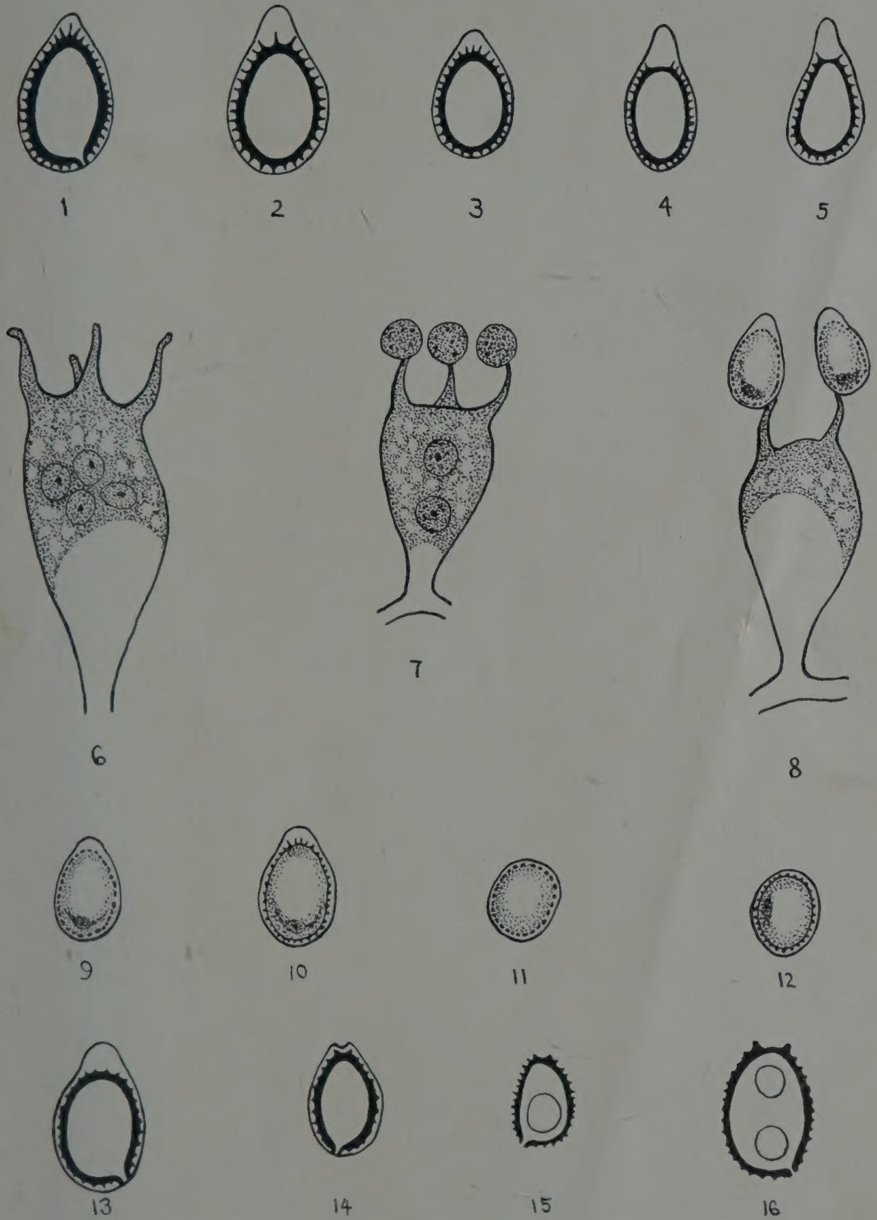
FIG. 12.—Transverse section of basidiospore of *G. applanatum*, showing same stage as in fig. 10.

FIG. 13.—Basidiospore of *G. applanatum* in longitudinal section, showing chitin reaction of the endospore; note swelling of both endospore and epispore, former giving a reddish violet color reaction.

FIG. 14.—Basidiospore of *G. applanatum* in longitudinal section, showing chitin reaction as in fig. 13; in this case hyaline beak has collapsed.

FIG. 15.—Basidiospore of *G. applanatum* in longitudinal section after having been heated in 20 per cent H_2SO_4 which has completely dissolved the epispore.

Fig. 16.—Basidiospore of *G. tornatum* in longitudinal section after being heated in 5 per cent H_2SO_4, giving same result as shown in fig. 15.



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